Combinatorial omics analysis reveals perturbed lysosomal homeostasis in collagen VII-deficient keratinocytes

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Supplemental Experimental Procedures

Cell culture and stable isotope labelling by amino acids in cell culture

Keratinocytes were isolated from skin samples of the eight patients and, as controls, from the skin of seven healthy, age-matched controls. Cells were subcultured and passaged in keratinocyte growth medium (Keratinocyte-SFM) (Invitrogen, Karlsruhe, Germany), supplemented with keratinocytes supplements (Bovine Pituitary Extract (BPE); EGF, human recombinant).

Normal human keratinocytes where seeded on 6-well-plate culture dishes. Medium was exchanged the following day and 10 ng/mL TGF-β 1 (Peprotech, Hamburg, Germany) was added. RDEB keratinocytes were incubated with 4.4 µg/mL TGF-β receptor type I and type II dual inhibitor LY2109761 (Adooq BioScience LLC, Irvine, CA, USA). Cells were harvested after 48 h by incubation with lysis buffer (25 mM Tris-HCL pH 7.5, 0.1 M NaCl, 1% NP-40, EDTA, Pefablock, Phosphatase inhibitor 3 cocktail, PHJ2) on a shaker for 20 min on ice. Cells were then scraped off, transferred into 1.5 mL tubes, vortexed and centrifuged at 13`000 rpm for 10 min. Supernatants were incubated for 5 min at 95°C in SDS-buffer and directly used for WB analysis or stored at -80°C until use.

For MS analysis, cells were cultured in keratinocyte growth medium (KGM2) (Promocell, Heidelberg, Germany) without arginine and lysine, but with “SupplementMix” (0.004 mL/mL bovine pituitary gland extract, 0.125 ng/mL recombinant human epidermal growth factor, 5 µg/mL

recombinant human insulin, 0.33 µg/mL hydrocortisone, 0.39 µg/mL epinephrine, 10 µg/mL human holo transferrin) and 0.06 mM $CaCl_2$, 210 mg/L L-arginine and 63 mg/L L-lysine for the unlabeled condition. Cells were "heavy" labeled for 2 weeks with 210 mg/L L-arginine-$^{13}C_6$-$^{15}N_4$ (Arg_{10}) and 63 mg/L L-lysine-$^{13}C_6$-$^{15}N_2$ (Lys_8) or "medium" labeled with 210 mg/L L-arginine-$^{13}C_6$ (Arg_6) and 63 mg/L L-lysine-$^{2}H_4$ (Lys_4). Biological replicates were obtained by swapping labels.

RNA isolation from primary human keratinocytes and RNAseq

Total RNA from 80-90% confluent keratinocytes was isolated using the RNeasy Mini Kit according to the manufacturer's instructions. Libraries were constructed with the stranded TruSeq GOLD protocol (RS-122-2301) followed by rRNA depletion (Illumina Ribo-Zero rRNA removal kit human, mouse, rat). Sequencing was performed on a HiSeq2500 (50 bp, Illumina, San Diego, USA). RNAseq raw data is available as a NCBI BioProject, ID PRJNA373827 (http://www.ncbi.nlm.nih.gov/bioproject/373827).

Functional annotation was done with a gene ontology analysis based on Cytoscape and ClueGO (2.2.4) (Bindea et al., 2009). Overrepresented GO terms from the categories "biological process", "cellular component" and "molecular function" were corrected by Benjamini–Hochberg procedure for multiple testing-controlled p-values. Significantly enriched terms were functionally grouped and visualized and the most significant term of each group was displayed.

Data analysis

Transcriptomics. RNAseq data were analyzed using the Freiburg instance of Galaxy (Afgan et al., 2016; Blankenberg et al., 2011). Therefore, a workflow was created including steps from quality-control of raw reads to calculation of differentially expressed genes. Quality control was performed using FastQC. After manual quality inspection, reads were trimmed with Trim Galore! accordingly. RNAseq data were mapped to the human (hg19) reference genome, using TopHat2 and the human gene annotation model (GRCh37.75) (Kim et al., 2013). After read mapping, htseq-

count was applied to count aligned reads per gene in a BAM file which overlap features in the gtf file (Anders et al., 2015). These count data then were used as input files for DESeq2 to calculate differentially expressed transcripts of RDEB patients compared to controls (Love et al., 2014). The results were filtered first by the adjusted p value 0.1 (FDR controlled with Benjamini-Hochberg procedure) and second by the log2 fold change. The workflow is publicly available on github https://github.com/galaxyproject/training-material/tree/master/RNA-Seq.

To show altered gene expression in RDEB keratinocytes compared to controls, cluster analysis was performed of 301 differentially regulated genes in RDEB keratinocytes (Benjamini–Hochberg corrected q-value 0.1) as determined by high-throughput sequencing of the transcripts. TPM values were log2-transformed and z-score normalized. Columns containing data from the different samples were hierarchically clustered and rows containing gene entries were clustered by k-means.

Functional annotation was done with a Gene Ontology (GO) analysis based on Cytoscape and ClueGO (2.2.4) (Bindea et al., 2009; Shannon et al., 2003). Over represented GO terms from the categories ‘biological process’, ‘cellular component’ and ‘molecular function’ were corrected by Benjamini–Hochberg procedure for multiple testing-controlled p-values. Significantly enriched terms were functionally grouped and visualized and the most significant term of each group was displayed.

Proteomics. To generate a list of extracellular proteins, the data were filtered using the freely available Perseus software to extract proteins that belong to the “matrisome” and/or are annotated as “extracellular” (extracellular region, extracellular region part, extracellular space, extracellular matrix, extracellular matrix part, proteinaceous extracellular matrix, extracellular organelle, extracellular membrane-bounded organelle, extracellular vesicular exosome) and “cell adhesion” (Tyanova et al., 2016). Data were readjusted to a median of 1 and log2 transformed and z-score normalized. SOTA clustering was performed of proteins quantified in intra- (11 clusters) and

extracellular (7 clusters) compartments of RDEB cells compared to controls. Proteins in each cluster were tested by DAVID for enriched GO terms (biological process (BP), cellular compartment (CC) and molecular function (MF)) applying the default settings with a minimum significance of $p<0.01$ (Huang da et al., 2009). Network analysis of significantly regulated ECM proteins (Welch's t-test, permutation-based FDR 0.05) was created by STRING DB (confidence score 0.4) and Cytoscape (Szklarczyk et al., 2015).

Immunofluorescence staining of frozen skin biopsy specimens

Immunofluorescence staining of RDEB and site-matched control skin was performed on 5 µm cryosections, which were air dried and incubated with primary antibodies at 4°C overnight. Primary antibodies to the following proteins were used: anti-collagen VII (LH7.2, Abcam), anti-S100A9 (AF2065, R&D Systems, Minneapolis, MN, USA) and anti-cathepsin B (clone 6D5 and 3E4) (Weber et al., 2015). After four washing steps of 5 minutes each, secondary antibodies were applied for 1 h. Secondary antibodies were: Alexa-488 anti-mouse IgG or Alexa-488 anti-rabbit IgG (Invitrogen, Darmstadt, Germany). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Millipore, Temecula, CA, USA). Stained sections were observed with an Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany). Images were captured using Zeiss internal software.

Active site labeling for cysteine cathepsins

Keratinocytes (10 cm^2 petri dishes, 80-90% confluent) were washed 3 times with ice-cold DPBS. Cells were lysed by adding 200 µL ice-cold sodium acetate buffer directly onto the petri dish. Cells were scraped off, incubated on ice for 30 min and centrifuged (10,000xg, 10 min, 4°C). Cell lysates (without protease inhibitors) were then used directly for activity assays to avoid degradation of proteins. Cysteine cathepsins were labelled by incubating cell lysates with DCG-04 (final concentration 10 µM) for 1 h on ice (Greenbaum et al., 2000). In order to distinguish western blot

signals, prior to DCG-04 treatment cell lysates were incubated for 15 min on ice with the cysteine protease inhibitor E-64 (final concentration 10 µM) or the specific cathepsin B inhibitor Ca074 (final concentration 5 µM). In order to reveal unspecific binding of the substrate, proteases were inactivated in a control sample through incubation for 10 min at 95°C prior to DCG-04 treatment. Afterwards, samples were directly boiled at 95°C for 10 min and then used for WB analysis or stored at -20°C.

Western blot

Cell lysate or ECM samples corresponding to 1/10 of confluent 10 cm^2 cell culture dishes were separated by SDS–PAGE using self-casted SDS gels and transferred onto nitrocellulose membrane. Membranes were blocked with 5% milk powder in 1x TBS-T for a minimum of 1 h at room temperature and incubated with primary antibodies diluted with the same buffer for 1 h or overnight at 4°C. HRP-conjugated secondary antibodies and a chemiluminescent detection assay (Immobilon Western, Millipore, Schwalbach, Germany) were used for visualization according to manufacturer's instructions.

For the active site labeling for cysteine cathepsins, fluorescently labeled streptavidin (LI-COR, Lincoln, NE, USA) was used to detect the biotin tag of the DCG-04 substrate. Detection was carried out on a Li-Cor Odyssey Infrared scanning system. *ß*-actin was used as loading control, using respective fluorescently labeled secondary antibodies.

To quantify changes of cathepsin B amount due to the treatment with TGF-β1 bands corresponding to the single-chain enzyme of four different controls were quantified using image J. To investigate changes of cathepsin B after treatment with LY2109761 bands corresponding to the single-chain enzyme of four different RDEB keratinocytes were quantified. Intensities (AU) were normalized to the β-tubulin signals from the same blots. Antibodies used for WB analysis: Anti-cathepsin B (3E4, Weber et al 2015) 1:1000; Anti-Phospho-Smad3 (phospho S423 + S425;

EP823Y; ab52903, Abcam, Cambridge, UK) 1:1000; Anti-Phospho-Smad2 (Ser465/467 (138D4) Cell Signalling).

Autophagy assay

10 cm cell culture dishes (approximately 50 % confluent) were washed with PBS once and then incubated with fresh medium including 20 nM concanamycin A for 2 h in order to block degradation of autopahgosomes. Cells were then harvested, lysed and used for western blot analysis (anti-LC3 (clone5F10, Nanotools, Teningen, Germany)).